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FOXP3 gene expression in a tuberculosis case contact study

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Summary

Regulatory T lymphocytes (T_{regs}) that express FOXP3 are involved in the beneficial attenuation of immunopathology, but are also implicated in down-regulation of protective responses to infection. Their role in tuberculosis (TB) is unknown. We classified 1272 healthy TB contacts according to their tuberculin skin test (TST) and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) results and 128 TB cases, and studied the expression of *FOXP3* and interleukin *(IL)-10* in blood samples. Compared to the uninfected contact group (TST^-, $ELISPOT^-$), we observed higher levels of *FOXP3* mRNA in blood from TB patients ($< 0{\cdot}001$), but *IL-10* expression was slightly lower ($P = 0{\cdot}04$). In contrast, *FOXP3* expression levels were significantly lower ($P = 0{\cdot}001$) in the recently infected contacts (TST^+, $ELISPOT^+$) but there was no difference for *IL-10* ($P = 0{\cdot}74$). We hypothesize that during early/subclinical TB, most of which will become latent, $FOXP3^+$ T_{regs} may be sequestered in the lungs, but when TB becomes progressive, FOXP3 reappears at increased levels in the periphery. While these findings do not reveal the role, beneficial or harmful, of T_{regs} in TB, they emphasize the probable importance of these cells.

Keywords: *FOXP3*, IL-10, T_{regs}, tuberculosis

Introduction

Forkhead box P3 (FOXP3) is a transcription factor associated with functional regulatory T cells, known as T_{regs}. Although recent studies have revealed many different types of T_{regs}, both naturally occurring and inducible, FOXP3 has been shown to have a direct role in inducing immunosuppression and has been identified as a good marker for cells with a suppressor function [1–4]. In humans, these cells were first thought to be specifically $CD4^+$ $CD25^{high}$ naturally occurring T_{regs}, but more recent studies have shown this not to be the case and FOXP3 is also expressed in other cells (such as $CD8^+$) with a suppressor function [5–7]. FOXP3 came to light in this context when defects in the gene were found in mice suffering from a complex syndrome, Scurfy, characterized by autoimmunity, allergic disorders and enteritis. A similar condition in humans, immune polyendocrinopathy enteropathy X-linked (IPEX), was found to also have a mutation in the *FOXP3* gene. Thus cells expressing *FOXP3* are involved in limiting immunopathology, and it has emerged subsequently that several chronic inflammatory disorders in humans can be accompanied by reduced T_{reg} function [8]. This beneficial anti-inflammatory role is also seen in several models of infectious disease [9]. However, there is convincing evidence from a wide variety of infections that T_{regs} can also have detrimental roles and impair immunity [10,11].

In tuberculosis (TB), either function of T_{regs} could be crucial. Immunity is associated with a T helper 1 (Th1) response, and excessive down-regulation of Th1 might lead to disease. On the other hand, the disease is characterized by destructive lung lesions and immunopathology that are mediated largely by the immune system. This might be due to inadequate regulation.

A recent study revealed that *FOXP3* is present at a higher level in the peripheral blood of TB patients than in the blood of controls [12]. Similarly, expression of *FOXP3* was increased at the site of disease, and removal of the $CD25^{high}$ cells caused some increase in the lymphoproliferative response to antigens of *Mycobacterium tuberculosis* (*M. tuberculosis*) *in vitro* [12]. Nevertheless, this study does not determine whether the $FOXP3^+$ T_{regs} were impairing beneficial effector responses or usefully limiting immunopathological ones.

In order to gain further insight into the role of FOXP3 in the relationship between *M. tuberculosis* and the host we have studied its expression in peripheral blood from TB cases and contacts in The Gambia.

Materials and methods

Participants

The Medical Research Council (MRC) TB case contact study (TBCC) recruits TB cases over 15 years of age and their household contacts, at least 6 months of age [13]. The median age of cases is 28 years and of household contacts 15 years. This nested study, within the TBCC cohort, was approved by the combined MRC/Gambia government ethics committee and written informed consent was obtained from each individual. In accordance with the Extended Programme of Immunization (EPI) schedule, first introduced in 1979, all Gambian babies are vaccinated with bacille Calmette–Guérin (BCG) at birth. Since 1980 the coverage rate in The Gambia has been 80–90% (WHO/UNICEF Review of National Immunization Coverage 1980–2005). Household contacts underwent a tuberculin skin test (TST; 2 tuberculin units) using purified protein derivative (PPD; RT23, Staten Serum Institute, Copenhagen, Denmark) and an HIV test. A positive TST result was defined as a mean induration of the two measured diameters of ≥ 10 mm. Human immunodeficiency virus (HIV) infection was identified by competitive enzyme-linked immunosorbent assays (ELISA; Wellcome Laboratories, Kent, UK) and Western blot analysis (Diagnostics Pasteur, Marnes-la-Coquette, France). All contacts with a positive TST, and those with symptoms, were offered a chest X-ray and/or sputum testing. TB cases were referred to the government clinic for free TB treatment [13]. HIV positive subjects were offered post-test counselling and became eligible for free anti-retroviral therapy. Whole blood was collected for interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays and in PAXgene tubes (PreAnalytiX, Qiagen Ltd, Crawley, UK) for mRNA expression analysis.

Laboratory procedures

ELISPOT assays were conducted as described previously [14]. In brief, sequential peptides (15 mers overlapping by 10) spanning the length of early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 were used in pools at 2·5 μg/ml (synthesized by ABC, Imperial College, London, UK) [13]. A response to one or more pools of peptides was recorded as positive. PPD was used at 10 μg/ml. The positive control was phytohaemaglutinin (PHA) (2·5 μg/ml; Sigma-Aldrich, Poole, UK). All antigens were tested in duplicate wells. Positive wells were predefined as containing at least 10 spot-forming units (SFU) more than, and at least twice as many as, negative control wells. Positive and negative control wells were required to meet set criteria defined previously [13]. ELISPOT plates were counted using an automated ELISPOT reader (AID-GmbH, Strassberg, Germany).

For reverse transcription–polymerase chain reaction (RT–PCR), total RNA was isolated from whole blood according to the manufacturer's instructions (PreAnalytiX; Qiagen Ltd) and reverse transcribed to cDNA using 1 μM oligo-dT_{15} (Sigma-Genosys, Cambridge, UK), 10 units ribonuclease inhibitor (Invitrogen Ltd, Paisley, UK) and following the instructions of the Omniscript RT Kit (Qiagen Ltd).

Gene expression profiles of *FOXP3* and *IL-10* were measured by RT–PCR using the Corbett Research Rotorgene 3000 (Sydney, Australia) with QuantiTect SYBR Green PCR kits (Qiagen Ltd). Specific sequences and final concentrations were as follows: *FOXP3* (0·4 μM forward primer: 5′-ACCTGGAAGAACGCCATC, 0·4 μM reverse primer: 5′-TGTTCGTCCATCCTCCTTTC), *IL-10* (0·4 μM forward primer: 5′-CTTTAATAAGCTCCAAGAGAAAGGC, 0·4 μM reverse primer: 5′-CAGATCCGATTTTGGAGACC). The data were expressed as copy numbers relative to the housekeeping gene, human acidic ribosomal protein (*HuPO*), as described previously and validated for use in TB [15]. Any sample with expression of *HuPO* < 10 000 copies/μl was excluded as an inadequate result and expression of *FOXP3* was not determined in this sample.

Comparisons

Because of the intense but unquantifiable background of exposure to environmental mycobacteria in The Gambia and the high sensitivity of the T cell response to PPD [16], we standardized our inclusion criteria to include only those with a positive PPD ELISPOT response. The reasons for a failure of cells from citizens of a mycobacterium-rich environment to recognize PPD are likely to be complex, may contribute to heterogeneity because of different environmental pressures and introduce too many confounders into a large immuno-epidemiological study such as this. Two subgroups of these PPD ELISPOT-positive donors were then selected: those with strong evidence of recent *M. tuberculosis* infection (TST ≥ 10 mm and ESAT-6/CFP-10-ELISPOT positive), referred to as double-positives (+/+), and those least likely to be recently *M. tuberculosis*-infected (TST = 0 mm, ESAT-6/CFP-10-ELISPOT negative), referred to as double-negatives (–/–).

Data management and analysis

All data were entered using double data-entry or by electronic transfer into a database and verified, as described previously [17]. The relative copy number of each gene was $\log_{10}$-transformed to normalize the data and a linear mixed model was used to compare the case contacts according to infection phenotype. A random effect for household was used to allow for household clustering and the fixed effects for proximity to case, age, gender and HIV status to adjust for possible confounding. A *t*-test was applied when comparing cases and contacts. All statistical analyses were conducted using STATA software (version 9·2; Stata Corp, College Station, TX, USA).

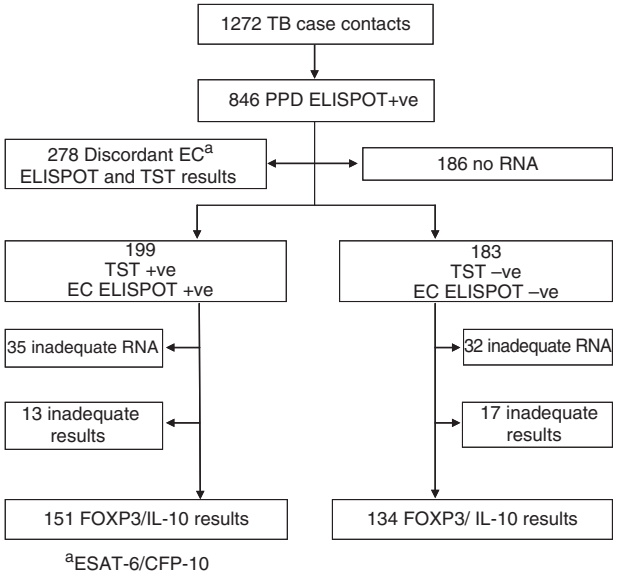

Fig. 1. Selection of tuberculosis (TB) case contacts and samples used for RNA analysis. Phenotype polarization of 1272 TB case contacts with enzyme-linked immunospot (ELISPOT) and tuberculin skin test (TST) results, to identify mycobacterially responsive individuals as double-positive (+/+), that have strong evidence of recent *Mycobacterium tuberculosis* infection [TST ≥ 10 mm and early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10-ELISPOT positive] and double-negative (–/–), those least likely to have recent *M. tuberculosis* infection (TST = 0 mm, ESAT-6/CFP-10-ELISPOT negative).

Results

From our case contact study we identified 1272 healthy contacts of sputum smear and culture-positive TB cases, recruited consecutively from 228 households between June 2002 to February 2004, who had ELISPOT and TST results (Fig. 1). One hundred and twenty-six TB cases were bled and had RNA available for the study; 114 had an HIV test: nine (8%) were positive. The median age of the cases was 27 years (mean 33 years, range 15–86). The median age of the case contacts was 15 years (mean 20 years); almost half were male (45·8%) and 2·2% of 1261 tested were HIV positive. Of the healthy contacts, 846 (66·5%) were PPD ELISPOT positive and were eligible for gene expression studies.

Table 1 shows the characteristics of the 382 contacts selected for RT–PCR. There were no significant differences between contacts classified as recently infected *versus* those classified as not recently infected by age, sex, ethnic group, BCG scar or HIV status.

Figure 2 also shows the RT–PCR results according to infection status with *M. tuberculosis*. *FOXP3* gene expression was significantly lower in those recently infected, compared with those not recently infected ($P = 0{\cdot}001$) (Fig. 2a). In the same comparison the *IL-10* gene transcript levels were not significantly different ($P = 0{\cdot}74$) (Fig. 2b). Because Guyot-Revol *et al.* have reported that expression of *FOXP3* is increased in the peripheral blood of TB patients [12], we compared the level of expression in TB cases ($n = 126$) with individuals within the double-negative group aged 15 years or over ($n = 67$). The *FOXP3* level in this double-negative subset did not differ significantly from the level in the whole group, but was significantly lower than expression in the TB cases ($P < 0{\cdot}001$) (Fig. 2c). There was a reduction, of borderline significance, of *IL-10* expression ($P = 0{\cdot}042$) between the double-negative case contacts group ($n = 65$; age > 15 years) and the cases ($n = 122$) (Fig. 2d).

Discussion

The study of patients and controls by Guyot-Revol and colleagues indicated that progressive TB is accompanied by increased expression of FOXP3, both in the blood and at the site of the disease [12]. Our data emphasize this point, because *FOXP3* levels were higher in the blood of patients than in the blood of either infected or uninfected contacts.

Table 1. Characteristics of tuberculosis (TB) case contacts who were purified protein derivative (PPD) enzyme-linked immunospot (ELISPOT) positive divided into subgroups of double-positives, those that have strong evidence of recent *Mycobacterium tuberculosis* infection (TST ≥ 10 mm and ESAT-6/CFP-10-ELISPOT positive) and double-negatives, those least likely to have recent *M. tuberculosis* infection (TST = 0 mm, ESAT-6/CFP-10-ELISPOT negative).

Characteristics	Double-positive ($n = 199$)	Double-negative ($n = 183$)	*P*-value
Mean (median : range) age (years)	20·4 (18; 0·5–80)	18·9 (15; 0·5–89)	0·38
Male, *n* (%)	88 (44·2)	76 (41·5)	0·60
Ethnic group, *n* (%)			
Mandinka	41 (20·6)	61 (33·3)	
Jola	53 (26·6)	39 (21·3)	
Wolof	32 (16·1)	31 (16·9)	
Fula	22 (11·1)	15 (8·2)	
Other	51 (25·6)	37 (20·2)	0·062
BCG scar present, n (%)	77 (38·7)	84 (45·9)	0·15
HIV positive, *n* (%)[a]	3 (1·5)	5 (2·7)	0·41

BCG: bacille Calmette–Guérin; HIV: human immunodeficiency virus; [a]$n = 380$ tested.

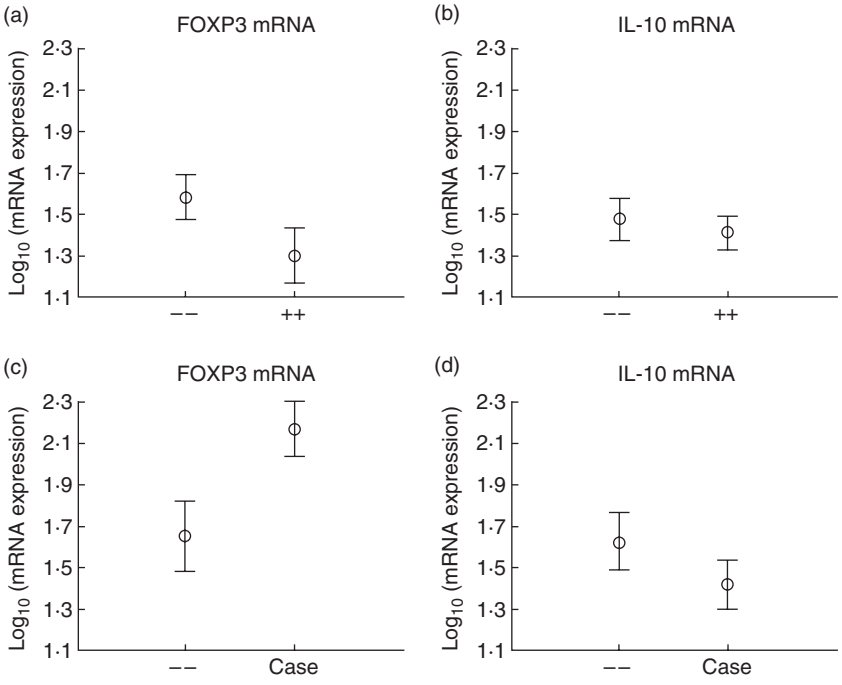

Fig. 2. Forkhead box P3 (FOXP3) and interleukin (IL)-10 expression in tuberculosis (TB) cases and contacts. (a) FOXP3 expression according to *Mycobacterium tuberculosis* infection status in TB case contacts, segregated according to their tuberculin test (TST) and enzyme-linked immunospot (ELISPOT) responses [+/+ TST ≥ 10 mm and early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10-ELISPOT positive; $n = 151$]; (–/–; TST = 0 mm, ESAT-6/CFP-10-ELISPOT negative; $n = 134$). (b) IL-10 expression according to *M. tuberculosis* infection status in TB case contacts, segregated as in (b). (c) FOXP3 expression in *M. tuberculosis* cases ($n = 126$) and contacts ≥ 15 years of age ($n = 67$) with no evidence of infection (–/–). (d) IL-10 expression in *M. tuberculosis* cases ($n = 122$) and contacts ≥ 15 years of age ($n = 65$) with no evidence of infection (–/–).

By contrast, our own study indicates that individuals who develop both positive (ESAT-6/CFP-10)-ELISPOT and TST after exposure to *M. tuberculosis*, and who are therefore probably infected but without overt disease, have low *FOXP3* expression in the peripheral blood compared to similarly exposed individuals who remain negative by these tests.

Considering the two sets of data together, one interpretation is that an excessive rise in *FOXP3* expression (presumably indicating increased T_{reg} activity) leads to progression, while a low expression of *FOXP3* correlates with a vigorous and effective response that maintains the infection in a non-progressing latent state. While this hypothesis is attractive, there are problems with it. If a positive (ESAT-6/CFP-10)-ELISPOT test tends to indicate the presence of living *M. tuberculosis* in the donor [18], then the most resistant individuals are those who remain ELISPOT negative despite exposure to an infectious case. Indeed, the ability of BCG vaccination to reduce the percentage of children that develop positive (ESAT-6/CFP-10)-ELISPOT responses following exposure to *M. tuberculosis* has been used as a measure of the protective efficacy of BCG [18]. Following this train of thought, we are forced to conclude that the double-negative contacts were the most resistant, because they showed no evidence of infection, but they also had more expression of FOXP3 than did the double-positive infected contact group. The dilemma can be resolved by suggesting that these individuals rapidly eliminated the organism without permitting sufficient replication to drive an IFN-γ response to ESAT-6 or CFP-10. By contrast, the double-positive contacts might have intermediate resistance to *M. tuberculosis*, and after exposure undergo transient infection that is controlled effectively and in most cases driven into latency. Our data are compatible with the view that, in this intermediate group, control of infection is effective partly because expression of *FOXP3* becomes reduced and so protective effector mechanisms increase.

However, recent work in HIV^+ individuals suggests another interpretation. The low blood *FOXP3* in the double-positive contacts might merely reflect sequestration of T_{regs} at the site of infection. In HIV infection *FOXP3* can be decreased in the blood but increased in the nodes, where viral replication takes place [19]. Therefore, if only the blood is examined, a low level of *FOXP3* can appear to correlate with high viral load [20], but in reality *FOXP3* levels might be very high in the lymph nodes [19]. In our study low blood *FOXP3* correlates with evidence of a high TB exposure (even though the infection is subclinical) because the TST and (ESAT-6/CFP-10)-ELISPOT are both positive in this subset of contacts. Therefore, by analogy with the situation in HIV, the T_{regs} might be sequestered in the infected lung.

There was no difference in *IL-10* expression in the blood of infected and uninfected contacts, despite the difference in *FOXP3* levels. However, there is an unresolved controversy as to whether $FOXP3^+$ T_{regs} themselves secrete the regulatory cytokines IL-10 and transforming growth factor (TGF)-β. There is evidence that they can cause their release by other cell populations, similar or identical to the inducible T_{regs}, Tr1 and Th3 [21]. Either way, it might be significant that peripheral blood lymphocytes from some severely ill patients release IL-10, rather than IL-2 or IFN-γ, in response to mycobacterial antigen *in vitro* [22]. Nevertheless, subclinical infection does not appear to affect blood levels of *IL-10* mRNA, although our results show a difference of borderline significance in *IL-10* expression between

TB cases and contacts without evidence of recent M. *tuberculosis* infection.

If the T_{regs}, but not the IL-10-secreting cells, are sequestered in the infected lung, is their function beneficial or detrimental? A recent publication suggests that in the presence of Toll-like receptor (TLR)-2 agonists (which are abundant in *M. tuberculosis*), T_{regs} are expanded but also inactivated, so that as long as the infection (and hence the TLR2 agonist) is present, they do not inhibit the necessary effector functions. Then, after the infection (and hence the TLR-2 agonist) has been cleared, the expanded population of T_{regs} regains function and terminates the inflammatory response [23]. Further studies will be required to test this hypothesis in relation to early subclinical TB.

This study has certain limitations. First, we did not assay FOXP3 in isolated $CD4^+$ T cells, or use another method such as flow cytometry to ascertain levels of T_{regs}. However, FOXP3 is not only expressed in $CD4^+$ T cells, and it is associated with regulation even when expressed in other cell types [5,6]. Therefore we consider that the highly significant changes in *FOXP3* expression in a large study are indicative of changes in regulatory activity. Secondly, for the reasons outlined in the Methods section, we studied only those tuberculosis contacts who were responsive to PPD in the PPD ELISPOT assay (66·5% of the donors). Consequently, our observations are limited to that subset of the contact population, but we point out that they do represent the majority. Finally, we identified individuals with recent infection using a dual phenotype; donors had to be both PPD skin test positive and ESAT-6/CFP-10 ELISPOT positive. This resulted in the exclusion of a substantial group of individuals who were positive in only one of these tests. We regard this exclusion as justified, as we cannot place these 'discordant' individuals definitively in the uninfected or recently infected categories, and these individuals will need to be studied separately in the future.

In conclusion, we show that individuals who become subclinically infected following exposure to *M. tuberculosis*, as indicated by both positive TST and positive (ESAT-6/CFP-10)-ELISPOT, have a lower expression level of *FOXP3* in their peripheral blood. We speculate that this is due to sequestration of T_{regs} at the site of infection in the lungs, but there was no ethical justification for attempting to prove this using bronchoalveolar lavage. Neither our data nor those of Guyot-Revol *et al*. cast any light on the role of T_{regs} in TB. Detrimental (anti-effector) and protective (anti-immunopathological) roles remain equally likely. These studies do indicate their relative importance, however, and set the stage for further detailed studies of T_{regs} in TB research.

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